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Ubiquitination assay

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An abbreviated version of this protocol was published in Nature Communications in Sep 2019

Modulation of M2 macrophage polarization by the crosstalk between Stat6 and Trim24

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Detailed protocol

Ubiquitination assay

1. Wash the cells with PBS and then resuspend the cells with PBS. Centrifuge at 500×g for 5 min at 4 ºC and remove the supernatant carefully.
2. Lyse the cells in 120 µl RIPA buffer (protease inhibitors and NEM should be added freshly just before starting lysing the cell) by pipetting up and down for 15-25 times, keep on ice for 30 min.
3. Centrifuge at 13000 rpm for 5 min at 4 ºC and collect the supernatant into a new tube.
4. Save 30 µl cell extract as input and then add 30 µl 2× loading buffer, boil at 100 ºC for 5 min.
5. The remaining 90 µl of the lysate is used for immunoprecipitation. Add 10 µl 10% SDS in the cell lysate to a final concentration of 1% SDS and boil at 100 ºC for 5 min. Then add 900 µl RIPA buffer to the final concentration of 0.1 % of SDS.
6. Add 20 µl protein-A/G-coupled agarose beads, keep on shaker for 1-2 hour at 4 ºC. Centrifuge at 7000 rpm for 2 min at 4 ºC. Transfer the pre-cleaned lysate into a new tube carefully. Add the relevant substrate proteins antibody and incubate overnight at 4 ºC.
7. Add 30 µl protein-A/G-coupled agarose beads and keep on shaker for 4 hours at 4 ºC.
8. Wash the beads with RIPA buffer containing protease inhibitors and NEM for 3 × 5 min, remove the supernatant and add 20 µl 1× loading buffer, boil the beads at 100 ºC for 10 min.
9. Load the sample and run SDS-PAGE and the immunoprecipitated samples are immunoblotted with anti-ubiquitin antibodies.

Solutions:

1. RIPA buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 1 mM EDTA
2. Protease inhibitors and NEM: PMSF (100 mM solution in isopropanol, 1/100 vol), DTT (1 M solution in 0.01 M sodium acetate (pH 5.2), 1/1000 vol), Aprotinin (10 mg/mL solution in 0.01 M HEPES (pH 8.0), 1/1000 vol), Pepstatin A (1 mg/mL solution in ethanol, 1/1000 vol), NEM (2 M NEM solution in ethanol, 1/500 vol)

Tested antibodies and agarose beads

Antibody/Agarose beads	Concentration	Company	Catalogue Number
Ub (P4D1)	1/1000	Santa Cruz	SC-8017
K63-linkage Specific Polyubiquitin (D7A11)	1/1000	Cell Signaling Technology	5621
Agarose beads		Santa Cruz	SC-2003

The ubiquitination of CBP

How to site:

1. Yu T, Gan S, Zhu Q, Dai D, Li N, Wang H, Chen X, Hou D, Wang Y, Pan Q, Xu J, Zhang X, Liu J, Pei S, Peng C, Wu P, Romano S, Mao C, Huang M, Zhu X, Shen K, Qin J, Xiao Y. Modulation of M2 macrophage polarization by the crosstalk between Stat6 and Trim24. Nat Commun. 2019 Sep 25;10(1):4353. doi: 10.1038/s41467-019-12384-2. PMID: 31554795; PMCID: PMC6761150.

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